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Sublethal Anesthesia of the Southern Pygmy Squid, *Idiosepius notoides* (Mollusca: Cephalopoda), and Its Use in Studying Digestive Lipid Droplets

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Abstract. We immersed southern pygmy squid, *Idiosepius notoides* Berry 1921 (hereafter squid), in three anesthetics (cold seawater, magnesium chloride [$MgCl_2$], and ethanol [EtOH]). Anesthesia was important for inducing both immobility and body transparency, conditions that were required for measuring extracellular lipid droplets. Cold anesthesia (4°C, day 1) led to frequent inking and some mortality. The $MgCl_2$ treatment (1–5%, day 18) did not induce body transparency and was often lethal. The 2% EtOH anesthesia (12–15°C, days 1–12) was most successful: it induced no inking and was followed by 100% recovery and postanesthetic survival (>7 days). There was no difference in induction times for squid anesthetized on day 1 versus day 2 after collection, with loss of body color patterns in ~12 sec followed by loss of mobility at ~30 sec. Time to immobility was related to time to transparency. Size (dorsal mantle length) was not related to time to induction or recovery. Although all squid survived three anesthesias with 2% EtOH, third inductions were significantly slower than first inductions. Anesthesia greatly improved accuracy of locating, counting, and measuring lipid droplets. With sublethal anesthesia, we detected extracellular lipid droplets in the digestive system of all 42 freshly collected squid. Without anesthesia, we failed to see cecal droplets in 20 of 41 squid that had them, and we failed to see digestive gland droplets in five of 23 squid that had them. Light EtOH anesthesia, including repeated treatments, did not seem to move droplets between the digestive cecum and the digestive gland or to induce expulsion of these droplets from the digestive tract.

INTRODUCTION

Cephalopods have complex nervous systems and may show their distress in a variety of ways, such as vigorous movements, skin color changes, and inking. Proper anesthesia is an important and appropriate procedure for calming these active molluscs before handling them for weighing, measuring, or surgical procedures (Moltschaniwskyj et al., 2007). Cephalopod anesthesia has been reported as at "a relatively primitive stage," even though anesthetics have been used for decades on these molluscs (Boyle, 1991).

Anesthesia is physiologically complex and not well understood for any organism, in part because not all anesthetics function the same way at the molecular level, e.g., they may bind into different sites on cellular proteins (Urban, 2002). Although anesthesia is medically defined as treatment that provides insensibility to pain (Urban & Bleckwenn, 2002), the term is often applied in the molluscan literature to any procedure that induces immobility, without reference to pain blockage; we use that latter meaning here.

According to Messenger et al. (1985), the first detailed study of cephalopod anesthesia was provided by Andrews &Tansey (1981). Cephalopod anesthesia typically is accomplished by immersion of the organism in seawater containing the anesthetic, sometimes with the intention of at least temporary survival of the animal (Andrews & Tansey, 1981; Boyle, 1991). Loss of swimming and righting ability, which typically occur before loss of breathing, are considered partial or light anesthesia (Messenger et al., 1985; Boyle, 1991). Loss of respiratory ventilations of the mantle cavity is a marker for induction of full or complete anesthesia (as defined by Young, 1971 in Andrews & Tansey, 1981; Boyle, 1991). Intentional overdoses (via either longer exposure to anesthetics or exposure to higher doses) are intended to humanely kill the cephalopod (e.g., before dissection), and they have been termed terminal anesthesia (Boyle, 1991), overanesthesia, and euthanasia (Moltschaniwskyj et al., 2007). As more humans begin to consider the ethical treatment of cephalopods (Mather & Anderson, 2007), clear information on efficacy of various anesthetics is warranted.

The best anesthetic for cephalopods seems to vary with species. Anesthetics tested on cephalopods include ethanol (EtOH), phenoxyethanol, urethane (ethyl carbamate), magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$), tricaine mesylate (MS-222), and cold water (as cited in Andrews & Tansey, 1981; Messenger et al., 1985; Moltschaniwskyj et al., 2007; Sen & Tanrikul, 2009). Andrews & Tansey (1981) showed that

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immersion in cold seawater (~4°C) was superior over the use of EtOH or urethane for relaxing *Octopus vulgaris*. Messenger et al. (1985) determined that $MgCl_2$ was superior to EtOH or urethane; although it was slightly slower, it seemed to be less traumatic on members of the five cephalopod genera they tested, including species of cuttlefish, squid, and octopods. In contrast, O'Dor et al. (1990) reported that EtOH was less stressful than $MgCl_2$ for producing light anesthesia in the squid species *Loligo pealei*, *Loligo opalescens*, and *Illex illecebrosus*. More recently, Berk et al. (2009) tested an ice bath and MS-222 in *L. pealei*, discarding both procedures after high distress and mortality and opting instead for rapid intubation without anesthesia.

The world's smallest squid (~0.5–3.0-cm mantle length) are placed in the family Idiosepiidae and include approximately a half dozen species. These Indo-Pacific cephalopods, despite their small size and lack of fishery importance, have recently become the topic of research (Boletzky, 2005), including analyses of nervous systems (Shigeno & Yamamoto, 2002), egg masses (Kasugai & Ikeda, 2003), life history traits (Tracey et al., 2003), extracellular lipid droplets (Eyster & van Camp, 2003), embryonic brains (Yamamoto et al., 2003), external digestion (Kasugai et al., 2004), and adhesive organ histochemistry (Byern et al., 2008).

During our initial study of yellowish, hydrophobic, sudanophilic spheres detected in the lumen of digestive organs of southern pygmy squid, *Idiosepius notoides* Berry 1921 (Eyster & van Camp, 2003; hereafter squid), we avoided chemical anesthesia because it might cause movement of material between adjacent digestive organs (Bidder, 1966). In that prior work, we confirmed the lipoid nature of these droplets; they are noteworthy because cephalopods apparently have limited capacity to metabolize or store lipids, relying instead on carbohydrate and protein metabolism for their energy sources (Hochachka et al., 1975; Storey & Storey, 1983; O'Dor & Webber, 1986). Although these droplets were detected in the lumen of digestive organs, it is unclear whether the droplets are metabolized, function in buoyancy, or are metabolic waste. We wanted to determine the frequency of occurrence of these small extracellular droplets and to follow their presence over time in live squid, so we needed a sublethal method of immobilizing the squid.

In cephalopods, including *Idiosepius* species, body color patterns are produced by muscles acting on pigment-containing sacs of the chromatophores (Boyle, 1991; Hanlon, 2010). Because our captive squid were often active and darkly pigmented (Figure 1), we needed a procedure that would not only immobilize the animals but also cause mantle tissue to relax to an almost transparent, uncolored state so that we could see through it to locate, count, and measure lipid droplets in live squid. In addition, we wanted an anesthetic procedure that was (1) quick and light (reducing anesthetic stress to the squid and allowing us to measure more droplets on the same day), (2) did not cause expulsion of the oil droplets out of the digestive system, (3) allowed the squid to recover normal swimming and feeding behavior after treatment, and (4) allowed posttreatment survival of at least 1 wk so that the squid and lipid droplets could be studied over that time. Here, we report successful responses of squid to immersions in EtOH, with additional notes on lipid droplets, development of anesthetic tolerance, and testing with $MgCl_2$ and with cold (4°C) seawater.

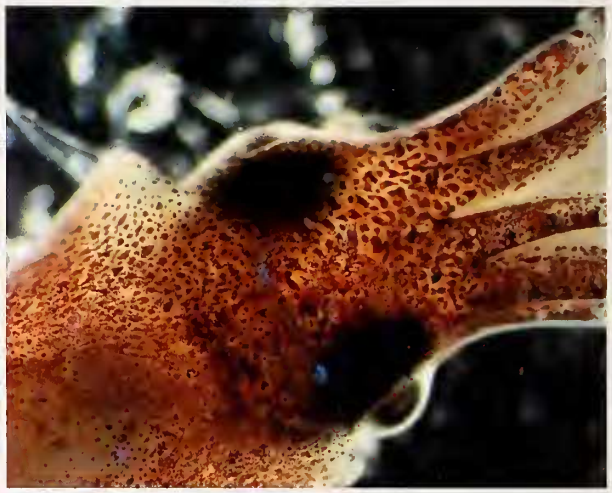


Figure 1. Head region of squid in seawater before anesthesia. Pigment-containing chromatophores are expanded, producing dark patterns; no papillae are noted. All squid shown in Figures 1–4, 7, and 12 were 6–9 mm in dorsal ML.

MATERIALS AND METHODS

Collection and Maintenance of Squid

Southern Pygmy squid are small (~2-cm dorsal mantle length) cephalopods that attach to seagrasses by use of glue glands located on the upper body (Norman, 2000). We collected these squid by seining over seagrass beds in several locations in South Australia.

For cold anesthesia tests, squid were collected near Myponga, South Australia, in the summertime (January 11, 2004) and acclimated to the laboratory at near field temperature (~22°C) for 1 day before testing. The rest of the squid in this study were collected at Noarlunga Reef, south of Adelaide, in the fall (April 21, 2002 [=day 0]). These squid were kept in an aquarium with recirculating seawater (~14–16°C; ~40 ppt salinity; 12:12-hr light:dark cycle) before and after testing.

To keep track of individual squid after testing began, we housed them singly in plastic screw cap straight-sided jars (~450 mL). To provide each squid with

flowing water, we replaced the central half of each lid and the entire bottom of each jar with plastic 2-mm gauge mesh. We then submerged the numbered jars on their sides in a flow-through tank attached to the recirculating seawater system.

We provided food to half the squid during week 1. Just before feeding time, all holding jars were cleaned out. Then, the holding jar of each fed squid was provided with 10 live field-collected mysid shrimp (daily through day 7). This feeding schedule was based on our videotaped observations that (1) the maximum number of mysids caught in one meal by isolated *I. notoides* before they stopped feeding was 10 and that (2) squid were more likely to feed when new food arrived; active but left-over shrimp frequently were ignored. In week 2, each squid was given five large store-bought brine shrimp daily (because poor weather prevented field collection of additional mysids). All squid in this work were starved on days of anesthesia before treatment.

Transfer and Immersion of Squid

On testing days, we transferred the holding jars from the aquarium building to the laboratory in large, water-filled trays. To reduce stress on the squid, we kept each squid submerged in seawater inside its holding jar until its testing time, and then we transferred it quickly to anesthetic solution (see below), returned it to fresh seawater as soon as measurements were completed, and maintained it in fluids between 12 and 16°C (except for 4°C treatments). We limited air exposures primarily because we observed that small air bubbles could become trapped in the mantle cavity and that these bubbles could resemble colorless oil droplets when viewed through the mantle.

To remove a squid from its holding jar, the jar was inverted (so that the removable lid was now on the bottom) and a glass dish (Petri dish) placed under the jar lid. As we lifted the jar out of the transport tray, water drained from the jar except for an ~1-cm-deep layer in the lid, held in by the glass dish. We unscrewed the lid from the jar and placed the glass dish (containing lid and squid) under a dissecting microscope for viewing. Previous testing showed that these shallow dishes were tall enough and held enough water to retain and submerge the squid.

Transfer of squid into anesthetic solutions (EtOH or $MgCl_2$) was accomplished by waiting until the squid swam over the central meshed portion of the jar lid. We then quickly lifted the lid out of the seawater, keeping the squid supported on the mesh grid of the lid. Next, we immediately inverted the lid over a small culture dish that was convexly full of anesthetic solution (~10 mL) so that the squid was immediately immersed in anesthetic solution. Compared to collecting and transferring each squid by small dip net or by hand, this rapid inversion–transfer method was faster and seemed less stressful for and damaging to the squid (based on absence of inking responses, absence of hyperactive swimming behavior, and subsequent health). If the squid did not instantly detach from the mesh, we gently squirted it with the same anesthetic solution until release. Exposure time was counted from the first second that the squid touched the anesthetic solution, even if the squid did not initially release from the mesh lid.

Anesthesia with Cold Seawater, EtOH, and $MgCl_2$

Cold seawater: On day 1 after collection, we transferred squid into cold seawater to determine whether the sudden temperature change from 22°C (room temperature) to 4°C would induce immobility, color change, movement of lipid droplets in the digestive system, or various combinations of responses. All squid were sexually mature (two females, eight males) and ranged from 6- to 19-mm dorsal mantle length (ML; mean = 11 mm). After lipid droplets became visible through the skin, the locations of the droplets were noted; locations were noted again at the end of the 60-sec immersion. Time to loss of mobility and time to loss of body color patterns were recorded as for EtOH immersions (see EtOH). After removal from the cold water, each squid was examined up to 4 min after treatment. No artificial ventilation was attempted if squid stopped breathing.

EtOH: The majority of our study involved 2% EtOH in filtered seawater (volume/volume) as the anesthetic. A few squid were exposed to 4% EtOH (see Repeated Anesthesia). The EtOH solutions were prepared fresh daily and used at 12–15°C to approximate the maintenance temperature; a solution was never reused. We recorded time (seconds) that we kept each squid immersed (=exposure time). Exposure time (123 ± 34 sec [mean ± SD]) was not constant because it varied with how long it took to measure each squid (ML), and then to locate, count, and measure visible lipid droplets (diameters).

For each squid immersed in 2% EtOH (or cold seawater), we recorded time to reach two induction markers: (1) complete loss of swimming (no jetting and no finning) and (2) complete loss of body color patterning (head and arms not included). These particular anesthetic stages were important for us because we needed stationary squid to make measurements and transparent squid to view the lipid droplets present in the digestive organs. We did not need any deeper anesthesia in this particular study, so we did not purposefully continue anesthesia until squid stopped respiratory ventilations.

Because newly collected cephalopods can be more susceptible to anesthesia than squid adapted to the laboratory (Messenger et al., 1985), we compared time to induction and time to recovery for squid anesthetized day 1 (24–30 hr after collection; n = 18) versus day 2 (43–47 hr after collection; n = 24). Average exposure times were ~2 min and were comparable on day 1 (2.2 min) and day 2 (1.9 min; unpaired *t*-test, $P = 0.12$).

$MgCl_2$: We immersed squid (n = 3) into 2.5–5% $MgCl_2$ in seawater (16°C, day 18). For one squid, $MgCl_2$ was added dropwise (1 drop/20 sec) to reach 1% at 10 min, followed by immersion for two more minutes. Solutions were prepared fresh before use, by mixing Merck hexahydrate ($MgCl_2 \cdot 6\ H_2O$) into distilled water and then mixing that solution with seawater (Messenger et al., 1985). After treatment, squid were moved to fresh seawater for recovery. If a squid stopped ventilating during the recovery phase, the mantle cavity was artificially flushed with seawater by using a pipette. Because these squid are so small (they are easily propelled around the culture dish when squirted by the pipette and cannot be easily held to administer artificial ventilations), when the upper body surface was sticky enough, a glass coverslip was attached to the squid; we held onto the coverslip to keep the opening of the mantle cavity oriented toward the pipette tip.

Impact of Anesthesia on Digestive Lipid Droplet Detection and Location

Bidder (1966) stated that anesthesia may cause movement of liquids between digestive organs of cephalopods. To address the question of whether light anesthesia moved these lumenal droplets of unknown function, we recorded the location of oil droplets at the beginning and end of the cold anesthesia tests as well as just before and then during 2% EtOH immersions. We used only newly collected squid (day 1or 2 after collection) to decrease other health issues that might impact outcome.

Just before the first EtOH immersion, we examined each animal microscopically (~×10) while it swam freely in its jar lid (sitting inside the glass dish); we hoped to locate lipid droplets visible during brief periods of squid inactivity that were accompanied by flashes of skin transparency. During the flashes, we also estimated the number of droplets at each location. To increase the number of squid we could test over a 24-hr period, we limited the preanesthesia microscopic viewing of each squid; if the mantle did not become transparent within 15 min, we proceeded to EtOH immersion. Each squid was carefully examined (~×10) while it was anesthetized, and the actual number of lipid droplets in each of three locations (cecum, left side of digestive gland, right side of digestive gland) was recorded and compared with that of the preanesthetic records. After measuring each squid (ML) and its lipid droplets (diameters), we immediately put it into a bucket (~8 L) of seawater to dilute and flush away the anesthetic. We later tested the impact of repeated anesthesia on the same squid (see Recovery from Anesthesia).

Recovery from Anesthesia

Timing of recovery from anesthesia began when treated squid entered fresh seawater. We defined full recovery as resumption of active swimming. Because preliminary work showed that (1) no squid resumed swimming in <60 sec after immersion in 2% EtOH ended and that (2) later during recovery, a squid sometimes looked immobilized (it had not moved) but it swam abruptly if gently prodded, we poked squid during their recovery phase. Beginning at 60 sec into recovery time, each squid was prodded gently with a glass rod once every 30 sec until it swam actively (either between proddings or at a prodding); to reduce stress on the squid, we did not prod more frequently.

Repeated Anesthesia: Second – Fifth Immersions

We hoped to track lipid droplets over time in individual squid to examine whether the droplets moved between organs, changed in volume, or were expelled from squid. Because such experiments required that squid be anesthetized more than once, we recorded squid responses to a variety of reanesthesia treatments.

The first, second, and third anesthesias all used 2% EtOH and occurred on days 1–2, 8, and 9–12 after collection, respectively. We compared three anesthetic markers for the first versus third EtOH anesthesias: (1) time to loss of swimming ability, (2) time to loss of body color patterns, and (3) time to full recovery. We also recorded location and number of lipid droplets (all anesthesias), volume of lipid droplets (first and third anesthesias), and squid survival rate (all anesthesias).

Because squid did not seem to become transparent as readily during third EtOH anesthesia, we raised the fourth EtOH treatment concentration to 4% for a few squid (n = 4). These four squid were immersed in 2% EtOH on days 1, 8, and 11 and then 4% EtOH on day 18. The fourth EtOH immersions lasted 1.5–2.5 min. No squid in our study was anesthetized more than five times or >3 wk after collection.

To compare the possible impact of nonconsecutive versus consecutive anesthesia, we exposed a few squid to each treatment pattern. Most reanesthesias were on nonconsecutive days, occurring three to four times over ~2.5 wk. For consecutive reanesthesia, squid (n = 4)

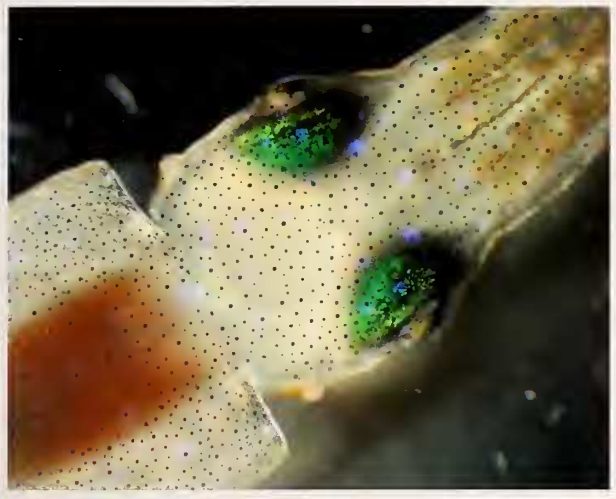


Figure 2. Head region of squid in postanesthesia recovery phase, showing minimized chromatophores, blue and green reflections from iridophores, and papillae in cheek areas.

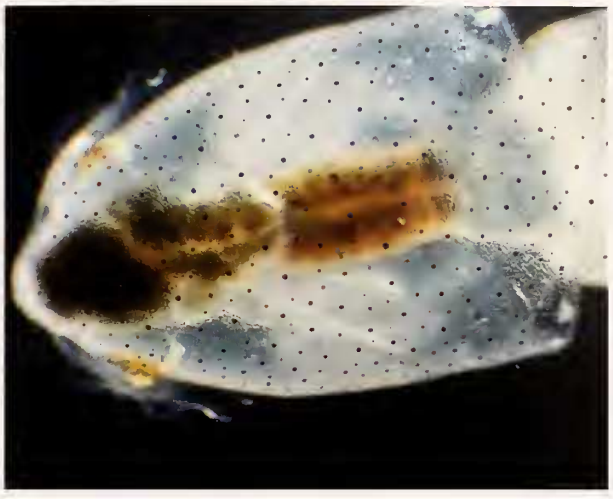


Figure 3. Body of EtOH-anesthetized squid, with transparent mantle that allows view of digestive organs.

were anesthetized first on day 1, fed mysids daily through day 7, and then reanesthetized daily on three to four consecutive days (days 8–10 or 8–11). On day 11, only one squid was reanesthetized; the other three squid looked unhealthy (i.e., less active, hypoventilating, or hyperventilating) and were sufficiently transparent to be examined without anesthesia. The four squid chosen for consecutive daily anesthesia were not selected at random but were chosen because on day 8 they were still very active and contained conspicuous lipid droplets. During consecutive anesthesia testing, the squid were housed individually between anesthesia treatments in glass bowls (100-mL static filtered seawater) so that we might detect lipid droplets if they were expelled. Aeration of cultures was avoided to avoid possible disruption of any expelled lipid into smaller droplets and also so that the surface of the water was more easily examined for oil droplets (Eyster & van Camp, 2003). Each bowl was cleaned, and the water was replaced daily with fresh aerated seawater at the same temperature.

Statistical Analyses

Data on squid dorsal MLs, induction times, exposure times, and recovery times were analyzed by unpaired *t*-tests, F-tests, Wilcoxon matched pairs tests, or Wilcoxon signed ranks tests.

RESULTS

Cold Seawater Treatment

Squid lost mantle color patterns very quickly after immersion in 4°C seawater, but they also inked frequently. Of the 10 squid tested, seven released ink, usually during the second half of their immersion minute. Squid in 4°C seawater lost their color patterns (in ~4 sec) before they stopped moving (in ~19 sec). After immersion, two squid with ink in the mantle cavity stopped breathing and died, so survival rate after these cold immersions was 80% (no artificial ventilation was attempted).

First EtOH Anesthesia (2% EtOH)

Body color patterns: The skin of untreated squid (Figure 1) had blackish and brownish patterns produced by chromatophore organs; blue and green colors were observed in untreated (Figure 1) as well as treated squid (Figure 2). The density of blackish and brownish colors in untreated squid (except during occasional flashes of transparency) prevented viewing of many lipid droplets located in the digestive system. However, in the first EtOH immersions, the squid quickly paled. In the time (~2 min) that it took to measure the squid and its lipid droplets, the most common skin response was transparency (Figure 3) over the entire squid body. Squid also produced a "bathroom-window translucency" (Figure 2), but the level of transparency shown in Figure 3 was sufficient and necessary to allow detection and measurement of lipid droplets in the digestive system.

Other color pattern changes involved some variation of paling. Some squid (three of 42) lost the black or brown patterns but turned opaque white (not transparent); compare the splotchy brown pattern on a white background in $MgCl_2$ (Figure 4).Three other squid paled when the color in the chromatophore sacs reduced to approximately half of their expanded surface area but did not minimize further. Some squid became transparent over most of the body but with

Figure 4. Body of squid in 3.75% $MgCl_2$, showing splotchy color pattern. The body is white but not transparent; some chromatophores are expanded and others are minimized.

colored patches remaining (i.e., they did not pale uniformly and completely). For example, in several squid, the ventral mantle became transparent, but the dorsal mantle between the fins did not become transparent or did not become transparent as quickly or as completely. Unfortunately, this was the key area that needed to be transparent for us to measure cecal oil droplets.

Time for the squid to develop skin transparency during the first EtOH immersion averaged 11–12 sec (range, 0.5–38 sec; Table 1). Time to reach transparency was equally quick for squid acclimated to the laboratory for 1 versus 2 days before anesthesia (Table; unpaired *t*-test, P = 0.893).

Time from immersion until the squid lost body color patterns (=time to transparency) was not related to squid size (Figure 5). That is, dorsal mantle length (range, 3.7–8.9 mm) was not related to time to reach this anesthetic state (F-test, $P = 0.598$).

No inking occurred in squid (n = 42) that were immersed in 2% EtOH at approximately the same temperature as their maintenance temperature and that were transferred by the rapid inverted lid method.

Movements: Untreated squid in small glass dishes without vegetation swam actively by jetting and finning, but they never jetted out of those dishes. Untreated squid never settled or crawled on the dish bottom, but they would attach underneath a floating piece of plain or marker-colored Parafilm® or plain glass coverslip (Figure 7). Treated squid soon lost mobility (Table) and rested on the dish bottom. No squid stopped respiratory ventilations or heart contractions during the first EtOH immersions.

Recently collected squid (day 1 or 2) continued swimming in 2% EtOH for approximately half a minute (33 ± 12 sec [mean ± SD]); only one squid kept swimming longer than 1 min (Figure 6). How long a squid swam in 2% EtOH was not related to squid size (Figure 6). Over the ML range of 3.7 to 8.3 mm, size was not related to time between immersion and immobility (F-test, $P = 0.92$).

Time to immobility was related to time to transparency (n = 37). The slope of the line in Figure 8 is significantly nonzero (F-test, $P = 0.0007$), suggesting that squid that took longer to lose body color also took longer to lose mobility.

Recovery: During recovery from the first EtOH anesthesia, only one squid stopped respiratory ventilations (apnea); ventilations paused for ~20 sec and then resumed without human intervention. Full recovery from the first EtOH anesthesia was therefore 100% (n = 42), followed by 100% postanesthetic survival of approximately a week (before the second EtOH anesthesia occurred on day 8).

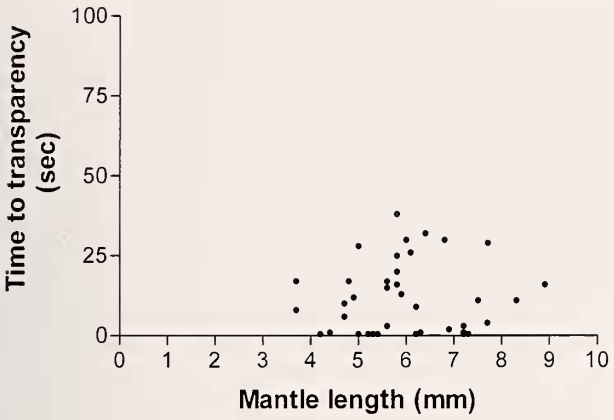
More than 20% of squid in first recovery (n = 9), regained dark pigmentation on the head while the body was still transparent. Before regaining mobility, four squid produced prominent papillae on the head, with one or two papillae near each eye, one near each "cheek" (Figure 2), or both. Although we did not measure time to recovery of body color patterns or

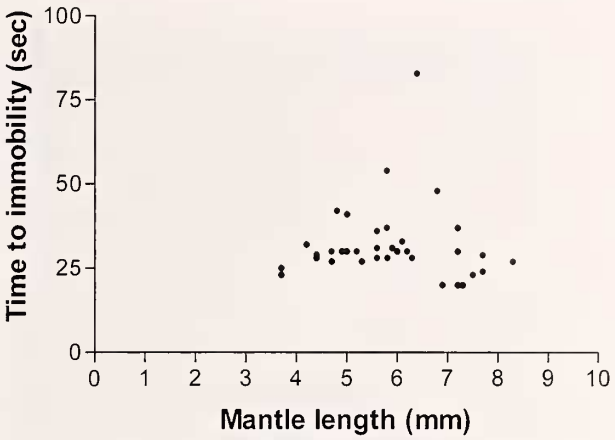
Table 1

Anesthetic induction times (including time to loss of body color patterns [transparency] and time to loss of swimming ability [immobilization]), exposure times, and postanesthesia recovery times for squid after the first (day 1 or 2) and third immersion in 2% EtOH (days 11–12), both at 12–15°C. Time values are averages.

Day after collection	Time to transparency (sec)	Time to immobilization (sec)	Exposure time (min)	Recovery time (min)	No. of squid
1	11.3	38	2.2	3.5	18
2	11.8	30	1.9	3.2	24
11 and 12	>90*	46	2.4	4.1	10

* Because most squid during third anesthesia never became fully transparent, time to transparency is given in this table as greater than the minimum exposure time.





Figures 5–6. Anesthetic induction of the squid during its first immersion in 2% EtOH. Squid size (as dorsal ML) was not related to anesthetic induction measured either as time (seconds) for squid to lose body color and become transparent (see Figure 5) or measured as time to lose swimming ability (see Figure 6). To aid comparison, data are graphed on the same scale for both anesthetic markers.

feeding ability, squid did regain apparently normal behaviors: they swam effectively, caught and ate arthropod prey, and produced and maintained skin color patterns during the week between the first and second anesthesias.

Most squid (71%) resumed active jetting and finning in 2–4 min (range, 1.3–7.5 min) after the first anesthesia (Figure 9). Time to recover swimming ability was not related to length of immersion in the anesthetic solution (range, ~1–4 min; F-test, $P = 0.57$). The first postanesthetic locomotion of four squid (including two papillated squid) was not by finning or jetting; these squid moved instead by walking on the dish bottom (by arm crawling).

Larger squid did not recover from anesthesia faster or slower than smaller squid over the range from 3.7 to 8.9 mm ML (Figure 9). Squid size (ML) was not related to time to full recovery of active swimming (F-test, $P = 0.632$).

For squid acclimated to the laboratory for 1 versus 2 days before the first anesthesia (Table), there was no difference in mean recovery times (unpaired t-test, $P = 0.443$). However, recovery times for day 1 squid were more variable; variances for day 1 versus day 2 were significantly different (F-test, $P = 0.009$).

Figure 7. Body of live, nonanesthetized squid attached to under surface of floating glass coverslip, showing retention of chromatophore expansion in the mantle region near the dorsal adhesive glands; the rest of the body has temporarily lost color and become somewhat transparent.

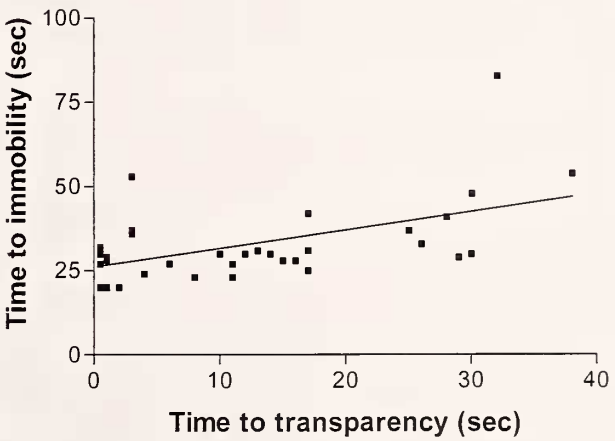


Figure 8. Relationship between time required for squid to lose body color (time to transparency) versus time to lose swimming ability (time to immobility) during first anesthesia with 2% EtOH days 1–2 after collection. The slope of the line is significantly nonzero (F-test, $P = 0.0007$).

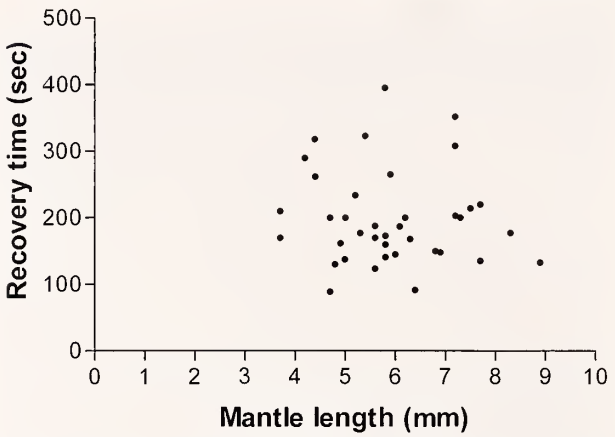


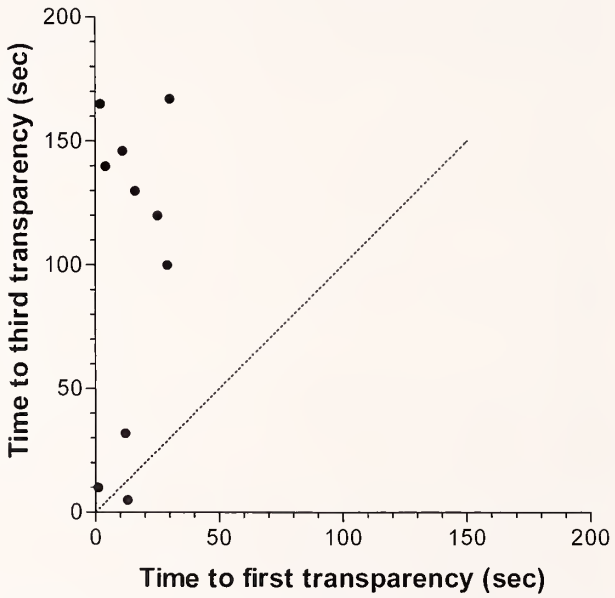
Figure 9. Squid body size (dorsal ML) versus time to recover swimming ability after first anesthesia (in 2% EtOH). Squid size (ML) was not related to time to full recovery (F-test, $P = 0.632$).

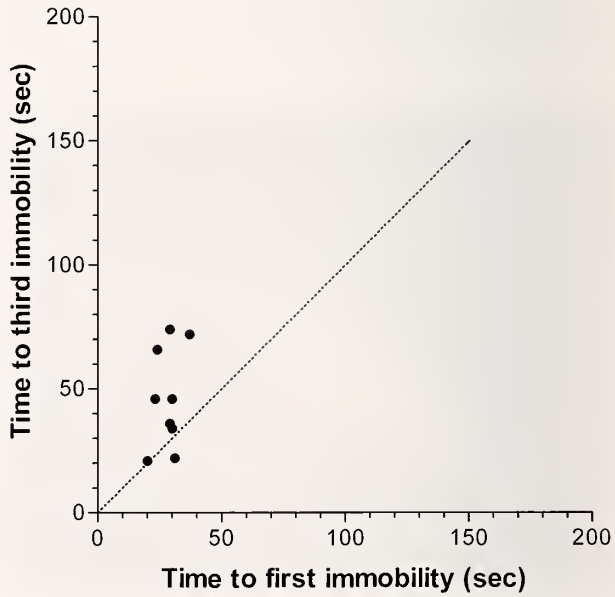
Repeated EtOH Anesthesias

Induction: Repeated EtOH anesthesias were similar in some ways and different in other ways from the first anesthesia (Table). Immersions in EtOH led to changes in body color pattern and mobility, but squid anesthetized more slowly during their third than first anesthesia (Figures 10, 11). For example, 90% of the squid (nine of 10) retained skin color patterns longer during their third EtOH immersion than they had during the first EtOH immersion (Figure 10). Time to body transparency for the third anesthesia averaged >90 sec compared with ~14 sec for first exposure for these same 10 squid (two-tailed Wilcoxon matched pairs test, $P = 0.004$). In the third anesthesia, seven of the 10 squid never became completely transparent during the time it took to measure them and to locate and measure visible lipid droplets. Consequently, the order of our two anesthetic induction markers was full body color loss before swimming loss in all squid (n = 42) during the first anesthesia and was reversed to swimming loss before full body color loss during the third anesthesia (n = 10). (Because transparency was not fully achieved in most squid during the third anesthesia, time to transparency for statistical testing and in Figure 10 was recorded as equal to the immersion time, a more conservative set of values.)

During the third EtOH anesthesia, squid took significantly longer to lose their swimming ability than they had during their first anesthesia (two-tailed Wilcoxon matched pairs test, $P = 0.027$). They could still swim ~46 sec after immersion during their third anesthesia compared with ~28 sec during their first anesthesia (Figure 11).

Recovery: In the first, second, and third anesthesias in 2% EtOH, squid immersed for a few minutes recovered in a few minutes. Recovery averaged 3.3 min after the first anesthesia and 4.1 min after third anesthesia





Figures 10–11. Comparison of times to reach two anesthetic induction markers during first anesthesia (2% EtOH, day 1 or 2) versus third anesthesia (day 11 or 12) for squid (n = 10). During the third anesthesia, squid took longer to lose body color patterns (see Figure 10) and longer to lose swimming ability (see Figure 11) than they had during the first anesthesia. All data above the dotted reference line represent slower inductions in the repeated anesthesias. To aid comparison of time to the two induction markers, data in Figures 10, 11 are graphed to the same scale.

(Table); these recovery times were not significantly different (two-tailed Wilcoxon matched pairs test, $P = 0.81$), but squid began to look less healthy after the third anesthesia.

No squid completely stopped respiratory ventilations during the first, second, or third immersion, but in recovery from the third anesthesia one squid was artificially ventilated with seawater for 8 min before it resumed breathing on its own. Survivorships for the first, second, and third EtOH anesthesias were 100%, with intervention on behalf of that one squid.

Fourth anesthesia: After it became apparent that squid seemed to be developing resistance to repeated 2% EtOH anesthesia, we tested some squid in a higher EtOH concentration and some in $MgCl_2$ solutions. Preliminary data (n = 4 squid) suggest that the fourth anesthesia (doubled to 4% EtOH) induced loss of skin color and loss of mobility comparably to the first anesthesia (using 2% EtOH), in that complete loss of body color patterns and loss of mobility occurred in both EtOH concentrations and occurred in that same order. Average time to transparency was 17 sec (cf. 12 sec in 2% EtOH, first anesthesia) and average time to immobility was 27 sec (cf. 30 sec in 2% EtOH, first anesthesia). Based on our small sample size, it seems that 4% EtOH as the anesthetic during the fourth anesthesia was faster at inducing color loss and immobility than was 2% EtOH during the third anesthesia. One squid died soon after the fourth anesthesia in 4% EtOH. The fourth anesthesia with $MgCl_2$ is described separately under $MgCl_2$ Treatment.

$MgCl_2$ Treatment

Squid (n = 4) immersed in $MgCl_2$ solutions (up to 12 min) did slow down but never became transparent throughout the mantle. In $MgCl_2$ solution, skin coloration became patchy, with the body sometimes transparent and sometimes white (Figure 4). Squid could still swim at 1 min after immersion in $MgCl_2$ solutions; they might rest on the dish bottom, but they would swim if they were gently prodded.

The one squid immersed in the highest $MgCl_2$ concentration (5%) showed an abnormal curved posture accompanied by jerky movements. Its mantle was not transparent and it retained a band of color mid-dorsally. Each tentacle displayed a prominent yellow stripe.

In 3.75% $MgCl_2$, the single squid tested had not become transparent at 8-min immersion, when it stopped respiratory ventilations. This squid was moved immediately to fresh seawater, and its mantle cavity was flushed using a pipette for 8 min before the squid ventilated on its own. It next hyperventilated (~100 times/min for several minutes). Ventilation slowed over the next 4 min and then stopped; the heart stopped and the body turned opaque white.

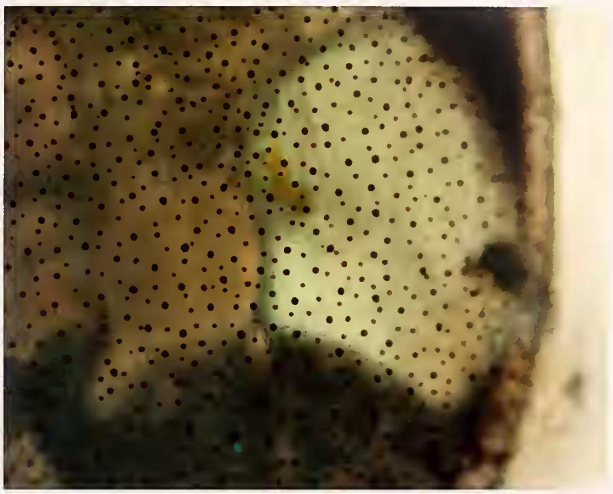


Figure 12. Cluster of droplets visible in the digestive cecum of a live, intact EtOH-anesthetized squid with retracted chromatophores and no colored body pattern. These dark yellow but small droplets were not detected several minutes earlier, before the squid was anesthetized.

In total, three of four squid stopped respiratory ventilations during or shortly after $MgCl_2$ treatment. None of these three squid survived the fourth anesthesia, despite up to 60 min of artificial ventilation per squid in fresh seawater. The sole squid that survived for several days after $MgCl_2$ treatment was the only squid exposed to $MgCl_2$ added dropwise (over a 10-min period to a final concentration of 1% $MgCl_2$, followed by 2 min in 1% $MgCl_2$). By 12 minutes, that squid was sluggish enough to be measured, but it was not fully immobilized. After 2 min recovering in fresh seawater, it began active finning and jetting and had a darkly pigmented mantle.

Impact of Anesthesia on Lipid Droplet Detection, Movement, and Retention

Without using anesthesia, we detected (during the 15-min allotted viewing time) extracellular lipid droplets in the digestive system in 25 of 42 live, intact squid on day 1 or 2 after collection. A few minutes later (per squid), using 2% EtOH anesthesia, we detected lipid droplets in all 42 squid. Comparatively large and dark yellowish droplets (see Eyster & van Camp, 2003:fig. 1a) were never missed in nonanesthetized squid. Droplets that we missed seeing tended to be comparatively small (Figure 12), pale yellow to colorless, or exhibited both traits. When we did not use anesthesia, we failed to detect droplets in both known locations: the digestive ceca and the digestive glands.

Just before the first EtOH anesthesia (days 1–2), lipid droplets were detected in the digestive cecum in 21 of 42 squid (50%). A few minutes later (per squid) cecal lipid droplets were seen in 41 of 42 anesthetized squid (98%), including all 21 squid that had visible cecal droplets before anesthesia. The one squid in which we did not detect cecal droplets (before or during its anesthesia) seemed healthy and was of average size (6.8 mm ML).

Just before the first EtOH anesthesia (days 1–2), lipid droplets were seen in the digestive gland in 18 of 42 squid (43%). In anesthetized squid, droplets were again detected in the digestive gland of those 18 squid plus five additional squid (55%).

In no case (on either day 1 or 2) were lipid droplets seen in a digestive organ just before anesthesia but then not found in that same organ during anesthesia. Also, no shifting of droplets between cecum and digestive gland was detected while the squid were immersed in the anesthetic solutions. Fluid seemed to flow from digestive gland to cecum in one anesthetized squid, but no droplets were seen in the digestive gland before or during the apparent flow.

Amongst squid that did have lipid droplets when anesthetized days 1–2 and that were re-examined ~1 wk later (second anesthesia), no starved individuals had any visible lipid droplets (n = 21) but all fed squid did (n = 20). In addition, the sum total lipid droplet volume detected in fed squid (in digestive gland plus digestive cecum) was greater on day 8 (after the second anesthesia) than the volume detected on days 1–2 (n = 20 squid; Wilcoxon signed ranks test, $P = 0.01$).

Total visible extracellular lipid droplet volume decreased in squid exposed to consecutive EtOH anesthesias (days 8–10 or 11) and starved during those days (Figure 13). Both cecal and digestive gland droplets were seen in all four squid on day 8 (during the second anesthesia), in three squid on day 9 during the third anesthesia, in two squid on day 10 (fourth anesthesia), and in one of the four squid on day 11. Lipid droplet volume decreased to zero or almost zero over several days of treatment, not after just one anesthesia event (Figure 13). No oil droplets were detected on the water surface in individual culture dishes.

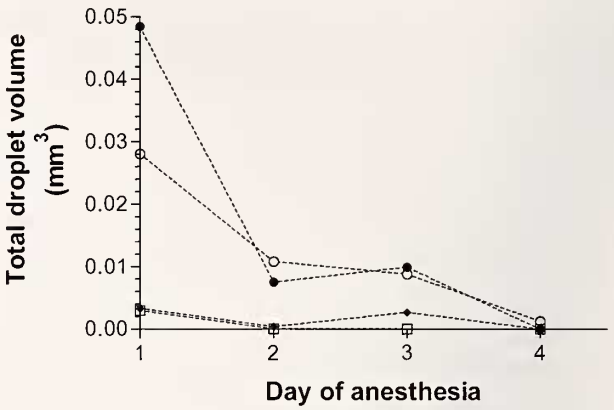


Figure 13. Extracellular lipid droplet volume (sum total volume of droplets in the digestive gland and the digestive cecum) in four squid exposed to starvation and repeated consecutive anesthesia administered 3–4 days in a row (with 2% EtOH, days 8–11 after collection).

DISCUSSION

Context for Using Chemical Anesthesia on Squid

In our previous work on extracellular lipid droplets in squid (Eyster & van Camp, 2003), we did not use chemical anesthesia for fear that it would induce movement of the digestive contents, as suggested by Bidder (1966). Instead, we placed squid in room temperature seawater into the freezer to the point of their immobility; then we decapitated them and cut them open to remove their lipid droplets. As our work continued and we began to examine lipid in numerous live squid over time, we needed a quicker and reliably sublethal method of repeatedly immobilizing the squid. Besides chilling, we tried other ways to observe a live squid microscopically in its relaxed, transparent state. For example, we confined individual squid under a small clear inverted dish about twice their length in diameter, but this caging method induced hyperactivity, and the duration of transparency did not last long enough for us to locate and count the oil droplets. Because captive squid typically spent many hours each day attached to the undersides of vegetation by their dorsal adhesive glands, we experimented with small floating pieces of transparent Parafilm and microscope coverslips (glass) in the observation dishes. In the absence of vegetation, nonanesthetized squid attached readily (in a few minutes) to the underside of these floating materials; after attaching, the animals often paled, allowing us to look for oil droplets in the digestive organs. Although this method avoided most stresses to the squid, the floating material jiggled as the squid ventilated, making accurate microscopic measurements difficult, and although the squid paled they were not always sufficiently translucent to allow viewing of the lipid droplets. While attached, the portion of the squid skin closest to the floating coverslip did not always pale as much as the rest of the squid (Figure 7) and transparency in that area of the body was critical for revealing all of the lipid droplets. Because waiting for a squid to pale enough on its own was very time-consuming and times of transparency were often too brief to allow detection of droplets, we finally used chemical anesthesia, as reported here. It was not our intention to conduct a thorough comparative study of anesthetic responses for

Southern Pygmy squid; we share a summary of the various responses of our squid to the anesthetics we used.

This is apparently the first description of sublethal anesthesia in idiosepiids, the world's smallest cephalopods. Anesthetic effects noted in our squid included (listed here in no particular order, without reference to order of induction, frequency of occurrence, or induction agent), (1) abnormal body posture, (2) suppression of jetting and finning movements, (3) paling of skin either in patches or to almost transparency, (4) decrease in mantle cavity ventilation rate, (5) cessation of respiratory ventilations, (6) inking, (7) loss of heart contractions, and (8) death. Previous studies have shown that side effects of cephalopod anesthesia include all of the above-mentioned effects as well as muscle contraction (with cold anesthesia), hyperactivity, hyperventilation, waves of skin color changes, attacks by other individuals, and attempts to escape (Hanlon et al., 1983; Messenger et al., 1985; Boyle, 1991).

Efficacy of EtOH as an Anesthetic

As Hanlon et al. (1983) and O'Dor et al. (1990) reported for other loliginid squid species, EtOH was a quick and effective light anesthetic for our squid. Time to both of our induction markers was rapid, typically occurring within ~0.5 min for first exposure to 2% EtOH in seawater. Stress from 2% EtOH immersion seemed low in squid based on normal body postures, lack of hyperactivity, lack of hyperventilation, rapid recovery times, quick return of feeding behavior, and absence of any inking in a large number of animals (n = 42). We do not know whether the respiratory movements seen in immobilized squid were effective or not, but even at the maximum exposure time (3.5 min), no squid stopped respiratory ventilations while submerged in 2% EtOH. We do not know whether anesthesia affected the digestive system, but treatment did not prevent recovered squid from capturing and eating prey or from surviving (with or without food) for 1 wk after treatment. In fact, fed squid ML grew an average of 0.4 mm during the week between their first and second EtOH anesthesias.

Although the present study shows that a few minutes immersion in 2% EtOH–seawater solution was an effective nonlethal anesthetic procedure for our squid, EtOH treatment can be traumatic or lethal for some cephalopods. For example, 60% of *Octopus vulgaris* hyperventilated and made escape attempts, and inking was common during 3–4-min exposures to 2% EtOH (Andrews & Tansey, 1981). EtOH (1.5%) was lethal for 20% of the squid *Illex illecebrosus* (Webber & O'Dor, 1986). For the congener *Idiosepius paradoxus*, 3% EtOH in seawater was used as an anesthetic before fixation for immunocytochemistry (Shigeno et al., 2008), but it is unclear whether those squid were anesthetized until loss of movement, loss of respiratory ventilations, or death. Even with very large cephalopods (e.g., cuttlefish up to 1 kg and octopods up to 9 kg; Zielinski et al., 2001), 2% EtOH solutions can be used for euthanasia.

Our two indicators of anesthetic induction (loss of ability to move and loss of ability to produce and sustain body color patterns) were relatively unambiguous markers with detectable end points. Because we exposed squid to EtOH only for as long as needed to measure their MLs and their visible microscopic oil droplets, we did not continue immersion until full anesthesia was reached; we assume that loss of respiration would be more stressful on the squid. An additional criterion to assess induction of anesthesia is whether the animal responds to the pinching of skin above the eye (Andrews & Tansey, 1981). However, because of the small size of our squid, eyelid pinches to assess anesthetic induction state were not attempted.

Of all induction and recovery information, data on time to loss of swimming ability were most closely clustered; almost half of the squid (20/42) stopped swimming between 27 and 32 sec after their first immersion in 2% EtOH in seawater. Recovery times were more variable than induction times, possibly in part because recovery was more difficult to assess. Because loss of ventilation was not used as an induction marker, resumption of ventilation could not be used for a recovery marker. It was apparent when the squid stopped swimming, but the point at which normal swimming behavior resumed or could have resumed was more subjective and reported values are subject to ±15-sec uncertainty. Also, some squid (15%) resumed movement by arm crawling before they resumed swimming. It is unclear whether these squid could have jetted as early as they crawled.

Compared with specimens acclimated to the laboratory, newly caught specimens of some cephalopods seem more susceptible to anesthesia, meaning that they suffer from more negative side effects. Therefore, cephalopods are usually held in the laboratory for a while before being tested. Twenty-four hours is considered an adequate acclimation period, but little information is available on this topic (Boyle, 1991). Here, we obtained no evidence that squid maintained in the laboratory for 2 days were less stressed during 2% EtOH anesthesia than were squid held for only 1 day before anesthesia. Thus, 1 day of acclimation before light EtOH anesthesia seems adequate for these squid.

Exposure time (i.e., how long it took us to measure the squid and its droplets) was 2.2 min on day 1 and 1.9 min on day 2. Although these times are not significantly different, the shorter time on the second

day of anesthetizing squid may be due to increased human efficiency rather than anything related to the squids' acclimation to the laboratory.

Repeated EtOH Anesthesia: Time to Induction, Order of Induction, and Health and Recovery

Squid often took longer to induce on subsequent EtOH immersions than during the first anesthesia, suggesting development of resistance to anesthesia, the specific anesthetic, or both. Our data do enable us to confirm whether the difference is due to repeated anesthetic events (previous anesthetic history), to change(s) in the squid during the time they were held in the laboratory, or both. For example, we did not compare first induction responses for day 1 versus day 8 of captivity. However, it seems more likely that squid would be less healthy after longer captivity and might therefore be less resistant, not more resistant, to induction, as shown here.

With the first EtOH anesthesias, loss of skin body color patterns in squid always preceded loss of swimming. With later EtOH exposures, this order was not maintained. For example, during the third anesthesia most squid lost mobility before reaching full transparency. Andrews & Tansey (1981) reported that order of induction events was the same in first urethane versus later urethane anesthesia (although the number of exposures is unclear).

Squid exposed to three EtOH anesthesias over 10 days seemed subjectively healthier than squid exposed to three EtOH anesthesias in three consecutive days; so, spacing of anesthesia may affect squid health, but we cannot rule out the impact of starvation that occurred on days of anesthesia. We knew from previous work (Eyster & van Camp, 2003) that some squid can survive without feeding for a couple weeks, but we have no data on impact of periodic starvation on their health. Squid (n = 4) maintained in a way similar to that of squid exposed to consecutive anesthesia in our study, but without anesthesia (i.e., in static culture in fingerbowls without food; Eyster & van Camp, 2003), looked healthier than anesthetized squid after 3 days, suggesting the repeated anesthesia did have a deleterious effect on the animals.

Despite some apparent development of resistance to EtOH anesthesia, as shown by changes in induction times and patterns, we consider reanesthesia with EtOH successful. Reanesthesia of cuttlefish with $MgCl_2$ was reported as successful (with full recovery; work of Kier et al., in preparation, as cited in Messenger et al., 1985), but repeated EtOH or urethane immersions were traumatic for octopus (Messenger et al., 1985).

Because our data suggest that anesthetized squid became resistant to induction by 2% EtOH, we performed one small trial test with 4% EtOH and one small trial with $MgCl_2$ (four squid each). Squid mortality in 4% EtOH was much higher (one of four squid) compared with that at 2% EtOH (zero of 42 squid); however, the squid exposed to 4% EtOH had been in the laboratory approximately 2 wk longer and thus were probably not as healthy. They also already had been exposed to 2% EtOH three times before their 4% EtOH treatment. Although induction times at 4% EtOH were similar to initial 2% EtOH induction, recovery times were more than twice as long, suggesting greater stress on the squid.

Cold Seawater and $MgCl_2$ Anesthesias

Although we had previously used chilled squid (Eyster & van Camp, 2003), we had only used lethal exposures (before incisions to remove droplets for testing). Although squid rarely inked when cooled slowly in a dish of room temperature seawater placed into the freezer, squid placed directly into 4°C seawater in the present work inked frequently (seven of 10). Based on our preliminary data, we cannot recommend sudden immersion in cold water for this squid, although retesting at other times of year may be worthwhile; perhaps the temperature drop from 22 to 4°C was too great and a smaller drop might be less stressful. Cold anesthesia may be ruled out for some cephalopods; for example, *Sepioteuthis lessoniana* should be kept at approximately 12°C during shipping (Ikeda et al., 2004). Cold anesthesia has been used successfully for 6.5-hr transports of the live squid *Todarodes pacificus*, although longer exposures (10–11-hr immersions) were fatal (Bower et al., 1999). Also, although cephalopods may survive cold anesthesia, it may be less effective in relaxing muscles (Andrews & Tansey, 1981).

$MgCl_2$ has been reported previously to be an effective anesthetic for a variety of cephalopods, including cuttlefish, squid, and octopods (Messenger et al., 1985). However, the smallest cephalopods they tested were 120 g or 50 mm ML, approximately 5 times longer than our squid and approximately 100 times more massive. Messenger et al. (1985) found $MgCl_2$ to have few traumatic side effects and to cause only one death (one of 17 animals) and only one inking. They also noted initial hyperventilation in all cephalopods; we did not note this particular side effect for squid immersed in $MgCl_2$.

Here, $MgCl_2$ was not as useful as EtOH for inducing minimized chromatophores in squid or for immobilizing the squid. Induction was slower than in EtOH, and these longer immersions were accompanied by loss of respiration that was followed by low percentage of recovery. Because we did not test response of recently collected squid to $MgCl_2$, we cannot rule out the possibility that this poor response was aggravated by

health of the squid (they had been in captivity ~19 days), previous anesthetic history (three previous EtOH anesthesias), or both. However, four other squid also held in the laboratory for ~19 days, and also previously exposed to EtOH anesthesia three times, suffered only 25% mortality in 4% EtOH compared with 75% mortality in $MgCl_2$.

Extracellular Lipid Droplets and Impact of Anesthesia and Reanesthesia

Eyster & van Camp (2003) reported extracellular lipid droplets in the digestive system in two freshly collected squid. Here, we expand that sample size and report droplets in all 42 freshly collected squid (examined days 1–2 without postcollection feeding).

We showed previously that these lipid droplets persisted in three squid starved for 7 days before sacrifice; squid starved longer had no detectable lipid droplets (Eyster & van Camp, 2003; Table). Here, we confirm loss of lipid drops by day 8 in starved squid, although squid were not checked for droplets on days 3–7. Although all starved squid (n = 21) had lost all visible droplets by day 8 and all fed squid (n = 20) contained visible droplets on day 8, we cannot determine whether those droplets demonstrate persistence of the original lipid observed on days 1–2, production of new lipid from mysid consumption, or both.

Anesthesia was important to our study of the lipid droplets in squid. Brief EtOH anesthesia greatly improved accuracy of data on location, number, and size of extracellular lipid droplets because it rapidly induced squid immobility and loss of mantle color patterns. For example, without anesthesia we failed to see cecal droplets in 20 of the 41 squid and digestive gland droplets in 5 of 23 squid that had them a couple minutes later when the squid were in a nonmobile, transparent, anesthetized state. We stated previously (Eyster & van Camp, 2003) that in cold-anesthetized squid, cecal droplets were easier to see than were digestive gland drops. This was because the cecum lumen was not obscured with dark material as was the digestive gland. Surprisingly, in the present study, we apparently missed cecal drops more frequently than digestive droplets when we tried to locate them without the aide of anesthesia.

We were concerned that anesthesia might induce these unusual extracellular oil droplets to move between organs, be expelled, or both (Bidder, 1966). However, we obtained no evidence that a single EtOH anesthetic event, anesthesia repeated on nonconsecutive days, or anesthesia repeated on consecutive days led to expulsion of these droplets from the digestive system. The fact that three of four squid still had lipid droplets during their third day of consecutive anesthesia suggests that light anesthesia did not lead to expulsion of droplets. We can rule out the idea that the lipid viewed on these days was formed from new meals because these four squid were not fed during anesthetic treatment days. It is unlikely that these squid expelled all of their old extracellular oil and made new oil droplets; preliminary work by Eyster & van Camp (2003) suggested that droplets can appear (in squid without apparent drops) approximately 3 hr after feeding.

It is harder to determine whether anesthesia induced any movement of oil drops between digestive ceca and digestive glands (than to determine whether drops were expelled) because both organ types already may contain these extracellular lipid droplets before anesthesia. This point is relevant to interest in where oil might possibly be stored versus possibly digested. Just before anesthesia, drops seemed to be in the same organ that they were found in during anesthesia. It is difficult to assess oil drop movement due to anesthesia because it is difficult to locate all drops without anesthesia, especially if the squid was too active or too darkly pigmented during the observation period or the drops were too small or colorless to be detected when the squid was moving slowly. Because the oil droplets can fuse or split, total droplet volume, not just droplet number, must be considered. Although data on repeated anesthesia 10–14 days after the first exposure do not suggest movement of droplets during anesthesia, we cannot rule out movement after recovery. Our data suggest that short exposures to 2% EtOH might not move these droplets between organs, but clear confirmation of organ-to-organ transfer of oil induced by anesthesia may require individual squid with digestive gland drops but no cecal drops, or vice versa.

Our work does not rule out catabolism of some of the oil, movement of some droplets between digestive organs, or expulsion of some small droplets from the squid, but it does seem to rule out EtOH-induced movement of all drops out of any particular digestive organ, or expulsion of all droplets after a single or even repeated immersion in 2% EtOH. This work clearly shows the advantage and safety of using EtOH anesthesia for calming and immobilizing Southern Pygmy squid.

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